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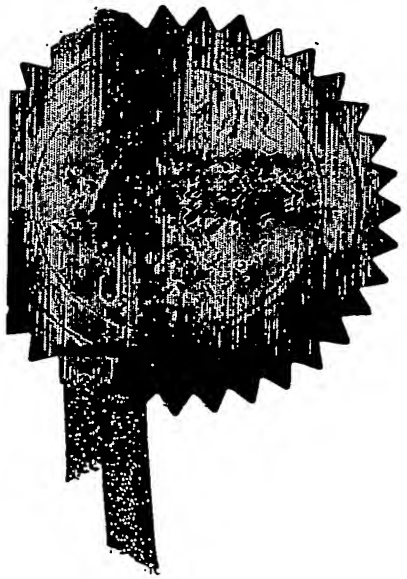

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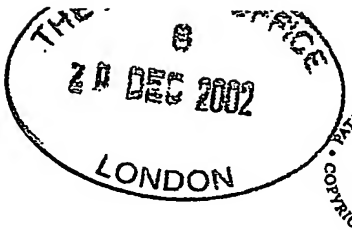

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1. Your reference 9224 GB/JSvn

2. Patent application number *(The Patent Office will fill in this part)* 0229832.1 — 20 DEC 2002

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

Axis-Shield Diagnostics Limited
The Technology Park
Dundee DD2 1XA
Scotland

Patents ADP number *(if you know it)*

If the applicant is a corporate body, give the country/state of its incorporation: Scotland

8531816001

4. Title of the invention

ACTIVATED FACTOR XII VARIANT

5. Name of your agent *(if you have one)* Abel & Imray

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

20 Red Lion Street
London
WC1R 4PQ
United Kingdom

Patents ADP number *(if you know it)* 174001

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Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*
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7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application

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Signature Abel & Imray Date 20/12/02

Abel & Imray

12. Name and daytime telephone number of person to contact in the United Kingdom — Judith Silveston — 020 7242 9984

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ACTIVATED FACTOR XII VARIANT

INTRODUCTION

The present invention relates to Factor XII, a component of the "contact activation system".

BACKGROUND OF THE INVENTION

Factor XII is an inactive zymogen present in normal blood. It is readily converted, in vitro, in the presence of kallikrein, high molecular weight kininogen and a negatively charged surface into a form, Factor XIIa, that is enzymatically active. *In vitro*, two forms of XIIa have previously been reported. The 80Kd form of the serine proteinase, often called Factor αXIIa, has a 52Kd heavy chain linked by a disulphide bond to a 28Kd light chain. Proteolysis of this factor releases a peptide from the heavy chain, and results in a product, Factor βXIIa, that retains serine protease activity, but in which the 28Kd chain of Factor αXIIa is disulphide-linked to a small peptide fragment derived from the former 52-Kd heavy chain. In many cases the small peptide fragment has a molecular weight of about 1000d, but fragments of different size have been observed.

WO90/08835 discloses an immunoassay for Factor XIIa. WO 90/08835 also discloses monoclonal antibodies 2/215 and 201/9, which bind to Factor XIIa, and methods for their production. Monoclonal antibody (mAb) 2/215 is produced by hybridoma 2/215, deposited at the European Collection of Animal Cell Cultures, Divisional of Biologics, PHLS Centre for Applied Microbiology and Research, Porton Down, Salisbury SP4 0JG, England (known as ECACC) on 16 January 1990 under the deposit number 90011606, and hybridoma 201/9, producing

monoclonal antibody 201/9, was deposited at ECACC on 18 January 1990 under deposit number 90012512.

Factor XIIa has long been known to be involved in the contact system of blood coagulation in vivo. More recent work indicates that Factor XIIa is also involved in other systems, including fibrinolysis, kininogensis, and also complement activation and angiogenesis. Many clinical and experimental data are accumulating to suggest that the contact system extends beyond haemocoagulation and that it has a role in maintaining vascular wholeness and blood pressure, that it influences various functions of endothelial cells and that it is involved in control of fibrinolysis and in maintaining the constitutive anticoagulant character of the intravascular space. Further clinical and experimental studies indicate that the contact system is involved in acute and chronic inflammation, shock of different aetiologies, diabetes, allergy, thrombo-haemorrhagic disorders including disseminated intravascular blood coagulation, and oncological diseases. Such conditions, include sepsis, spontaneous abortion and thromboembolism. In addition, Factor XIIa may be involved in tissue defence and repair. Yarovaya et al. (Yarovaya, G.A., Blokhina, T.B. & Neshkova,E.A. Contact system. New concepts on activation mechanisms and bioregulatory functions.Biochemistry (Mosc): 2002 Jan;67(1):13-24) is a recent review of the contact system and new concepts on activation mechanisms and bioregulatory functions.

SUMMARY OF THE INVENTION

The present invention is based on our surprising observation that some activated Factor XII (Factor XIIa) is associated with lipoproteins in the blood, and that measurement of this

lipid bound Factor XIIa provides information relating to a variety of clinical conditions.

The present invention provides a method for detecting or determining lipid bound activated Factor XII in a sample comprising tissue or a body fluid, for example blood, plasma or serum, obtained from a mammalian subject, generally a human.

The present invention provides a monoclonal antibody that is capable of binding to lipid bound activated Factor XII, for example, a monoclonal antibody having the same or similar properties as mAb 2/215 in relation to binding to Factor XIIa, but not including mAb 2/215 itself.

The present invention also provides a method for producing a monoclonal antibody that binds to lipid bound activated Factor XII, which comprises raising monoclonal antibodies against Factor XII or a fragment thereof and screening the antibodies against lipid bound activated Factor XII.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows lipid bound XIIa levels obtained for 12 healthy volunteers, assessed by addition of radiolabelled 2/215 antibody fragments to citrated plasma, removal of cellular material, precipitation of lipoproteins using a manganese/heparin precipitation method, and measuring radioactivity in the precipitated fraction.

Figure 2 shows lipid bound XIIa levels obtained for 64 patients admitted to hospital with chest pain, assessed by addition of radiolabelled 2/215 antibody fragments to whole blood, following removal of cellular material, precipitation of lipoproteins using a phosphotungstate precipitation

method, and measuring radioactivity in the precipitated fraction.

Figure 3 shows lipid bound XIIa levels (expressed as absorbance at 550 nm), as assessed by an ELISA method, obtained for 8 volunteers.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a method which comprises detecting or determining lipid bound activated Factor XII in a sample comprising tissue or, especially a body fluid obtained from a mammalian subject, generally a human.

The term "antibody" as used herein includes any antibody fragment that is capable of binding antigen, for example, Fab and $F(ab')_2$ fragments, and also recombinant, chimeric and humanized antibodies.

Measurement of lipid bound activated Factor XII may be performed on a sample of a body fluid, for example, whole blood or plasma.

Methods of carrying out immunoassays are well known, see for example, Methods in Enzymology, H. Van Vunakis and J. J. Langone (Eds), 1981, 72(B); Practice and Theory of Enzyme Immunoassays, P Tijssen, Laboratory Techniques in Biochemistry and Molecular Biology, R. J. Burden and P. H. Van Knippenberg (Eds), Elsevier, 1985; Introduction to Radioimmunoassay and Related Techniques, T. Chard, ibid, 3rd Edition, 1987; and Methods in Enzymology, H. Van Vunakis and J. J. Langone (Eds) 1981, 74(C).

Immunoassay techniques, both qualitative and quantitative, include ELISA (enzyme linked immunosorbent assays), Western

blotting, fluid phase precipitation assays, coated particle assays, competitive assays, sandwich assays, including forward, reverse and simultaneous sandwich assays, and solid phase radio immunoassays (SPRIA).

In one ELISA format that may be used according to the present invention, a capture antibody, especially a monoclonal antibody, that is capable of binding to lipid bound activated Factor XII, is immobilized on a solid phase support, for example, on a plastic or other polymeric material, for example on the wells of plastic microtitre plates, or on beads or particles, for example, as used in proprietary systems, for example, the IMx system of Abbott Laboratories, Abbott Park, Illinois USA. Samples comprising mammalian body fluids are incubated in contact with the immobilised capture antibody and any resulting captured activated Factor XII species are detected using a labeled antibody that is capable of binding to lipid bound activated Factor XII.

In another ELISA format that may be used according to the present invention, a capture antibody, especially a monoclonal antibody, that is capable of binding to an antigenic site that is not derived from Factor XII on lipoproteins, is immobilized on a solid phase support, for example, on a plastic or other polymeric material, for example on the wells of plastic microtitre plates, or on beads or particles, for example, as used in proprietary systems, for example, the IMx system of Abbott Laboratories, Abbott Park, Illinois USA. Samples comprising mammalian body fluids are incubated in contact with the immobilised capture antibody and any lipid bound activated Factor XII species are detected using a labeled antibody that is capable of binding to lipid bound activated Factor XII.

Another assay format that may be used according to the present invention is addition of a labelled antibody to the sample to be tested for lipid bound activated Factor XII. The lipoprotein fraction of the sample can then be separated from the remainder of the sample by any suitable technique, for example chemical precipitation of lipids using reagents such as manganese/heparin or phosphotungstate. Various methods are described in Demacker, P.N.M. *et al.* Clinical Chemistry Vol. 43, No. 4, 1997, p 663-668 and in Sharma, A. *et al.* Clinical Chemistry, Vol. 36, No. 3, 1990, p 529-532.

The labelled antibody may be polyclonal or monoclonal. Anti-human antibodies, for example, anti-human polyclonal antibodies, are often convenient for use as labelled antibodies. The label may be detectable directly or indirectly. Any appropriate radioisotope may be used as a directly detectable label, for example a β-emitter or an γ-emitter, examples being ^{125}I, ^{131}I, ^{3}H, and ^{14}C. For commercial use, non-radioactive labels, generally enzyme labels, are preferred. Enzyme labels are detectable indirectly. An enzyme label is, for example, alkaline phosphatase or a peroxidase, for example, horse radish peroxidase. An appropriate substrate for the chosen enzyme, for example, a substrate that gives rise to a detectable optical or fluorescence change, for example, phenolphthalein monophosphate or a fluorescent substrate, for example, methyl umbeliferone, is used. Alternatively, there may be used an enzyme reaction that can be followed using an electrochemical method.

Activated Factor XII, that is labeled, for example, radiolabelled or enzyme-labelled, may be used in a competitive assay for measurement of lipid bound activated Factor XII.

An example of an immunoassay for Factor XIIa is that described in WO90/08835. To determine lipid bound Factor XIIa it is recommended that mAb 2/215 is used . A different antibody, for example, a polyclonal antibody or a different monoclonal antibody may be used for detection.

Further methods utilise direct detection of a resulting antibody-antigen complex. Examples of such techniques are Surface Plasmon Resonance, Surface Acoustic Wave and Quartz Crystal Microbalance methodologies (Suzuki M, Ozawa F, Sugimoto W, Aso S. Anal Bioanal Chem 372:301-4, 2002; Pearson JE, Kane JW, Petraki-Kallioti I, Gill A, Vadgama P. J Immunol Methods ;221:87-94, 1998; Weisch W, Klein C, von Schickfus M, Hunklinger S. Anal Chem 1996 68:2000-4, 1996; Chou SF, Hsu WL, Hwang JM, Chen CY. Clin Chem 48:913-8, 2002).

A standard suitable for an assay for detection or determination of lipid bound activated Factor XII typically comprises a solution containing known amounts of lipid bound activated Factor XII. Alternatively, a standard may comprise activated Factor XII bound to a non-lipid supporting material, for example, a solid phase, or an aqueous solution of Factor XIIa may be used as a standard.

Determination of lipid bound activated Factor XII, may be performed by measuring its enzyme activity using a chromogenic substrate for example, as described by Vinazzer H., Thromb Res., 14, 155-66, 1979. This may involve a stage where one or more species are isolated from other species, for example, lipid bound Factor XII is separated from other species of Factor XII by means of an immunological separation using antibodies that show preferential binding for lipid bound activated Factor XII, followed by measurement of

enzymic activity in either the bound or un-bound fractions. Another example, is the separation of lipid bound activated Factor XII on the basis of its physical properties, for example separation on the basis of molecular weight using chromato-graphic procedures, followed by assessment of the enzymic activity of the separated material.

The invention, especially the immunoassays described above, provides a method of determination of lipid bound activated Factor XII, that can be used readily on automated equipment for large scale use.

The presence of lipid bound Factor XII or a fragment thereof, for example, lipid bound Factor XIIa, in a tissue sample may be detected using an immunohistological technique. For example, a monoclonal antibody as described above, labeled with an appropriate label, for example, a fluorescent label, may be used.

Monoclonal antibodies and immunoassays according to the present invention may be used in studies of coagulation systems and of thrombotic and other disorders, see also below.

The present invention further provides a kit for carrying out an immunoassay of the present invention, which kit comprises, each in a separate container or otherwise compartmentalised: (i) a monoclonal antibody that is capable of binding to lipid bound activated Factor XII, for example, mAb 2/215 or another monoclonal antibody having the same or similar Factor XIIa binding properties as mAb 2/215, and (ii) a labeled antibody capable of binding to lipid bound activated Factor XII when lipid bound activated

Factor XII is bound to the monoclonal antibody defined in (i).

The kit may comprise further components for carrying out an immunoassay, for example, as described above. The monoclonal antibody may be immobilised on a solid support.

A kit according to the invention may comprise, for example,

a) a monoclonal antibody that is capable of binding to lipid bound activated Factor XII, for example, mAb 2/215 or another monoclonal antibody having the same or similar Factor XIIa binding properties as mAb 2/215,

(b) a standard typically comprising of a solution containing known amounts of lipid bound activated Factor XII, activated Factor XII bound to a supporting surface or aqueous activated Factor XII.

(c) labelled antibody capable of reacting with lipid bound activated Factor XII when lipid bound activated Factor XII is bound to the monoclonal antibody defined in (i).

Alternatively, a kit may comprise labeled species of activated Factor XII, for use in a competitive assay.

A kit may also comprise further components, each in a separate container, for example, diluent(s), wash reagent solution(s)and substrate solution(s).

The present invention also provides an assay device suitable for carrying out an assay of the invention. The term "assay device" is used herein to denote means for carrying out an immunoassay comprising a solid phase, generally a laminar solid phase, for example, a membrane, sheet, strip, coating, film or other laminar means, on which is immobilized an

appropriate capture antibodies. The immobilized antibody is preferably present in a defined zone, called herein the "antigen capture zone".

An assay device may incorporate the solid phase within a rigid support or a housing, which may also comprise some or all of the reagents required for carrying out an assay. Sample is generally applied to an assay device at a predetermined sample application zone, for example, by pouring or dripping the sample on the zone, or by dipping the relevant part of the device into the sample. If the sample application zone is at a different site from the antibody capture zone, the arrangement of the device is generally such that antibodies in the sample migrate to the antibody capture zone. The required reagents are then applied in the appropriate order at designated application zones, which may or may not be the same as the sample application zone. Again, if the or any reagent application zone is at a different site from the antibody capture zone, the arrangement of a device is generally such that the reagent(s) migrate to the antibody capture zone, where any antigen-antibody complex formed is detected. All or some of the reagents required for an immunoassay may be incorporated within a device, in liquid or dry form. If so, a device is generally arranged such that interactions between different parts of the device, which interactions may occur automatically during the operation of the device or may be brought about by the user of the device, bring the various reagents into contact with one another in the correct sequence for the immunoassay to be carried out.

A wide variety of assay devices are described in the literature of immunoassays. Examples of membrane devices are described in U.S. Patents Nos. 4,623,461 and 4,693,984.

Depending on their design and their speed of action, some assay devices are called "dipsticks" and some are called "rapid assay" devices. A "rapid assay" device generally provides a result within ten minutes of the application of sample. (A typical microtitre plate or bead assay requires incubation steps, and generally takes at least an hour to provide a result.) Accordingly, although assay devices are generally more expensive than microtitre or bead format assays, they have particular uses in clinical testing, for example, when a result is required rapidly, for example, in the case of emergency surgery.

Assay devices have the particular advantage that they can be used without the need for sophisticated laboratory facilities or even without the need for any laboratory facilities. They may therefore be used for "on the spot" testing, for example, in an emergency room, in a doctor's surgery, in a pharmacy or, in certain cases, for home testing. They are particularly useful in territories where laboratory facilities are few and far between.

Factor XII and its activated form, Factor XIIa, are involved in blood coagulation and other contact systems, also known as contact phase systems, for example, fibrinolysis, complement cascade, inflammation and vasodilation, see Jacobsen S. and Kriz M., Br J Pharmacol., 29, 25-36, 1967; Kurachi K et al, Biochemistry, 19, 1330-8 1980; Radcliffe R et al, Blood, 50, 611-7, 1977; Ghebrehiwet B et al, J Clin Invest,71, 1450-6. 1983; Z Toossi et al, Proc Natl Acad Sci USA, , 89, 11969-72, 1992; Wachtfogel YT et al, Blood 67, 1731-7, 1986; Wachtfogel YT et al, Thromb Haemost, 80, 686-91, 1998; and Schreiber et al AD, J Clin Invest. ,52, 1402-9, 1973.

As Factor XII and its activated form, Factor XIIa are involved in haemocoagulation and have a role in maintaining vascular wholeness and blood pressure, in influencing various functions of endothelial cells, in control of fibrinolysis and in maintaining the constitutive anticoagulant character of the intravascular space, measurement of lipid-bound Factor XIIa is useful in investigations of those systems, including for example, fibrinolysis, complement cascade, inflammation and vasodilation. Clinical and experimental studies indicate that the contact system, which includes Factor XIIa, is involved in acute and chronic inflammation, shock of different aetiologies, diabetes, allergy, thrombo-haemorrhagic disorders including disseminated intravascular blood coagulation, oncological diseases, cardiovascular conditions, (for example, myocardial infaction, angina and acute coronary syndrome), angiogenesis, sepsis, spontaneous abortion and thromboembolism.

Determination of lipid bound activated Factor XII, are therefore useful in clinical and scientific investigations of such conditions, including diagnosing, predicting susceptibility to, monitoring and monitoring treatment of disorders where the contact system is involved, including acute and chronic inflammation, shock of different aetiologies, diabetes, allergy, thrombo-haemorrhagic disorders including disseminated intravascular blood coagulation and thromboembolism, oncological diseases, cardiovascular cardiovascular conditions, for example, myocardial infarction, angina, acute coronary syndrome, angiogenesis, sepsis, and spontaneous abortion.

Detection or lipid bound activated Factor XII, is therefore useful as an aid to diagnosing or monitoring diseases and

disorders in which the amount of lipid bound activated Factor XII is different from that in healthy subjects. Changes in the level of lipid bound activated Factor XII may be indicative of any of the conditions mentioned above. Changes in level in a subject with time may be indicative of change in the condition, for example, exacerbation of the condition, or improvement, for example, in response to therapy. Such methods of diagnosis and monitoring are part of the present invention.

The present invention provides a monoclonal antibody that is capable of binding to lipid bound activated Factor XII, for example, a monoclonal antibody having the same or similar properties as mAb 2/215 in relation to binding to Factor XIIa, but not including mAb 2/215 itself.

A monoclonal antibody that is capable of binding to lipid bound activated Factor XII, for example, a monoclonal antibody having the same or similar properties as mAb 2/215 in relation to binding to Factor XIIa, may be produced by methods that are known per se. Resulting antibodies are screened for those having the desired characteristics.

It may be useful to use monoclonal antibody 2/215 as a reference antibody in the screens for antibodies that bind to lipid bound Factor XIIa. A selected antibody may have binding characteristics for lipid-bound Factor XIIa that are the same as or similar to those of mAb 2/215.

The antigen used to raise the antibodies is Factor XII or a fragment thereof. An antigenic fragment of Factor XII may itself be immunogenic or may be too small to be immunogenic, in which case it may be converted into an immunogen, for example, by conjugation to another peptide, for example, as

described below. The term "an antigenic fragment of Factor XII" as used herein includes both a fragment, for example, a peptide, and an immunogenic form of such a fragment if it is not itself immunogenic.

An antigenic fragment of Factor XII may be Factor XIIa, for example, Factor α-XII or Factor β-XIIa or a fragment thereof, for example, a peptide that is a fragment of Factor βXIIa that is or that includes at least one antigenic determinant capable of recognising anti-Factor βXIIa.

Methods of preparing immunogens are known to those in the art. Any of these methods may be utilised to render immunogenic or to improve the immunogenicity of Factor XII or antigenic fragment thereof, see also WO90/08835.

For example, Factor βXIIa may be used as the immunogen to raise anti-Factor XIIa monoclonal or polyclonal antibodies. Factor βXIIa may be produced by a method which comprises first isolating Factor XII from fresh or freshly frozen plasma, for example, using a combination of ammonium sulphate precipitation and anion exchange chromatography for example, according to the method described by K. Fujikawa and E. W. Davie (Methods in Enzymol, 1981, 80, 198-211). Methods for converting Factor XII to Factor βXIIa and isolating Factor βXIIa from the resulting mixture are described by K. Fujikawa and B. A. McMullen (Journal of Biol. Chem., 1983, 258, 10924-10933) and B. A. McMullen and K. Fujikawa (Journal of Biol. Chem. 1985, 260, 5328). To obtain Factor βXIIa, Factor XII is generally subjected to limited cleavage, for example, by chemical or enzymatic digestion, for example, using trypsin or a trypsin-like enzyme, generally in a highly diluted form, for example, in a molar ratio of trypsin:Factor XII of 1:500,

for example, in a weight ratio trypsin:Factor XII of 1:75 and the cleavage products separated, generally by chromatography.

An antigenic fragment of Factor βXIIa may be produced by degradation of Factor βXIIa by enzymatic or chemical means. For example the disulphide-linked light chain peptide of Factor βXIIa can be obtained by reduction and carboxymethylation of Factor βXIIa and isolation of the fragment by chromatography (K. Fujikawa and B. A. McMullen Journal of Biol. Chem. 1983, 258, 10924). Alternatively, an antigenic fragment of Factor βXIIa may be produced if its amino acid sequence is known, synthetically, as may Factor βXIIa itself. Any of the many known chemical methods of peptide synthesis may be used, especially those utilising automated apparatus.

An antigenic fragment of Factor βXIIa may be produced using the techniques of recombinant DNA technology, as may Factor βXIIa itself. Cool et al, 1985 and 1987, loc. cit. have characterised a human blood coagulation Factor XII cDNA and gene. Recombinant production may be achieved by known methods, see for example, WO90/08835.

Unless specified otherwise, the terms "Factor βXIIa" and "βXIIa" as used herein include antigenic fragments of the Factor βXIIa molecule.

A monoclonal antibody for use according to the present invention must be capable of binding lipid bound Factor XII or a fragment thereof, for example, Factor XIIa. For example, it may be capable of binding to lipid bound Factor αXIIa, that is to say, it may be capable of recognising an antigenic determinant characteristic of αXIIa, or it may be capable of binding to lipid bound fragments of αXIIa, for

example, βXIIa. An immunoassay using an appropriate antigen may be used to determine the specificity of the antibody.

If desired, a monoclonal antibody for use according to the present invention may bind to both lipid bound Factor XII and lipid-bound Factor XIIa, or may bind to lipid bound Factor XIIa but show no significant binding to lipid bound Factor XII. In the latter case, the corrected cross-reactivity with Factor XII is, for example, 0.1% or less. A factor to take into consideration in assessing the cross-reactivity of an antibody of the invention with Factor XII is that even "pure" Factor XII preparations are almost inevitably contaminated with small amounts of Factor XIIa (Silverberg and Kaplan, Blood 60, 1982, 64-70). WO90/08835 gives details of methods of assessing the corrected cross-reactivity with Factor XII. Unless specified otherwise, the term "cross reactivity" is used herein to mean the corrected cross reactivity.

Methods used to produce monoclonal antibodies are well known, see for example, Methods in Enzymology, H. Van Vunakis and J. J. Longone (Eds) 1981, 72(B) and ibid, 1983 92(E).

Monoclonal antibodies may be produced, for example, by a modification of the method of Kohler and Milstein (G. Kohler and C. Milstein, Nature, 1975, 256, 495): female Balb/C or C57/BI0 mice are immunised by intraperitoneal injection of Factor XII or an antigenic fragment thereof, for example, from 10 to 30 μg, generally 20 μg of Factor βXIIa or a corresponding amount of the other antigen. The Factor βXIIa or other antigen is preferably conjugated to another protein molecule, for example, to a purified protein derivative of tuberculin or, preferably, to bovine thyroglobulin. The conjugation may be carried out, for example, by a carbodiimide method or by using a hetero-bifunctional

reagent. The immunogen is generally presented in an adjuvant, preferably complete Freunds adjuvant. This procedure is generally repeated at intervals, generally using the same immunogen in the same dose, for example, at 3 week intervals the mice are boosted with 20 μg of conjugated Factor βXIIa in complete Freunds adjuvant until suitable response levels are observed. A pre-fusion boost is preferably given prior to sacrifice, for example, intravenously 3 days prior to sacrifice.

The antibody response is monitored, for example, by RIA antisera curve analysis using, for example, ^{125}I radiolabelled Factor XII or a fragment thereof, for example, radiolabelled Factor βXIIa or another Factor βXIIa antigen prepared by the chloramine-T method (P. J. McConahey and F. J. Dixon, Int. Arch. Allergy Appl. Immunol, 1966, 29, 185). Purity is confirmed, for example, by using autoradiography, for example, of SDS-PAGE gels run under reducing conditions.

Immune mouse spleen cells are then fused with myeloma cells, for example, NSO mouse myeloma cells, for example in the presence of 40-50% PEG 4,000 or 50% PEG 1500. The cells are then seeded in wells of culture plates and grown on a selective medium. The supernatants are tested for reactivity against the corresponding purified Factor XII antigen, for example, in the case of a Factor βXIIa antigen, purified Factor βXIIa or other βXIIa antigen, for example, by a solid phase enzyme immunoassay, for example, using peroxidase-labelled anti-mouse IgG. All wells showing specificity for the antigen used for testing are generally taken for further secondary screening. The secondary screening consists, for example, of screening all specific antibodies for binding in solution to the appropriate antigen, for example, in the case of a Factor βXIIa antigen, Factor βXIIa or a Factor βXIIa

antigenic fragment that has been radiolabelled. These are preferably titrated to determine the antibody dilution required for 50% B max. Dose-response curves against cold, that is to say non-labelled antigen are generated, and are preferably also generated against Factor XII (if no cross-reactivity with Factor XII is desired), plasmin and fibronectin. The extent of cross reaction may be determined according to the following formula:

$$\frac{\text{Weight of Cold Standard Antigen to Achieve 50\% B max}}{\text{Weight of Cross-Reactant to achieve 50\% B max}} \times 100$$

Those antibodies showing an appropriate level of binding to the desired antigen, Factor βXIIa, for example, having affinity constants of at least $10^{10}M^{-1}$ are generally taken forward for cloning.

Successful clones are generally isotyped. The cells are then preferably sub-cloned by limiting dilution and again screened, generally using an enzyme immunoassay, for the production of antibodies to the desired antigen, for example Factor βXIIa. A selected sub-clone from each cloning may also be evaluated with respect to specificity and dose response using a radioimmunoassay or ELISA.

The antibodies may be screened for those showing a pre-determined apparent cross reactivity to Factor XII, preferably of 1.5% or less, for example 1% or less, for example 0.5% or less, for example, 0.1% or less.

As indicated above, screening against Factor XIIa is generally carried out first, but the two or optionally three screens may be carried out in any order.

Scatchard analysis may be done on the dose-response data to produce values for the affinity constants for each antibody.

Sub-cloned or cloned hybridoma cells may be injected intra-peritoneally into Balb/C mice for the production of ascitic fluid. The immunoglobulin may be precipitated from ascitic fluid, for example, at 4°C using saturated ammonium sulphate solution (equal volume). The precipitate is preferably purified, for example, it may be centrifuged, dissolved, for example, in 50mM Tris-HCl buffer pH 7.5 (volume equal to original ascites volume) and then dialysed against the same buffer. The immunoglobulin fraction may then be further purified by anion exchange chromatography, for example, the protein solution may be applied to a Mono-Q anion exchange column (Pharmacia) and eluted using a salt gradient in the same buffer according to the manufacturer's recommendations. The fractions containing immunoglobulin are generally pooled and frozen at -20°C for storage. Alternatively, hybridoma cells may be grown in culture for antibody production and the antibody isolated essentially as described above for ascites fluid.

Although the hybridomas described herein are derived from mouse spleen cells, the invention is not limited to hybridomas of murine or part-murine origin. Both fusion partners (spleen cells and myelomas) may be obtained from any suitable animal. Recombinant antibodies may be produced. Antibodies may be brought into chimeric or humanized form, if desired. The hybridomas are preferably cultured in vitro.

The present invention also provides polyclonal antibodies, also called a polyclonal antiserum, that are capable of reacting with lipid bound activated Factor XII. Such

antibodies may be labeled and used for detection of lipid bound activated Factor XII in an ELISA.

The invention also provides a method for the production of such a polyclonal antiserum, which comprises administering Factor XII or a fragment thereof, for example, Factor XIIa, especially Factor βXIIa to an animal, obtaining serum from the animal, screening the serum for binding to lipid bound activated Factor XII.

The following non-limiting Examples illustrate the present invention.

EXAMPLES

Example 1

In this example the existence of lipid bound activated Factor XII in plasma was demonstrated by addition to blood plasma of monoclonal antibody 2/215 antibody fragments labelled with a radiotracer (Iodine 125), precipitating the lipoproteins, and assessing the amount of radioactivity associated with the precipitated lipoprotein fraction.

Fab antibody Fragments of antibody 2/215 were prepared using an "Immunopure Fab Preparation Kit" (Pierce, 3747 N Meridian Road, PO Box 117, Rockford, IL 61105) according to the manufacturer's instructions. These Fab fragments were then radiolabelled with Iodine 125 by Amersham Pharmacia Biotech(Pollards Wood, Nightingales Lane, Chalfont St Giles, HP8 4SP United Kingdom).

Citrated plasma was obtained from 12 healthy volunteers (6 male and six female).

1 μl of radiolabelled antibody was added to 1ml of plasma from each of the volunteers. After incubation for 4 hours, the plasma was centrifuged at 12,000g for 10 minutes to remove cellular components. lipoproteins were precipitated by the addition to 400 μl of plasma supernatant of 300 μl of a precipitating reagent containing 500 mM NaCl, 215 mM $MnCl_2$ and 500 U/ml Heparin,. After mixing, and incubating for 10 minutes samples were centrifuged at 12,000g for 10 minutes. The supernatant was removed, and the lipoprotein pellet was washed (to remove any residual aqueous phase activated Factor XII), by resuspending the pellet in 1ml of the precipitation reagent, centrifuging at 12,000g for 10 minutes and removing the supernatant. After performing this wash procedure three times, radioactivity associated with the pelleted material was measured using a multi-well scintillation counter.

Figure 1 shows the lipid bound XIIa levels obtained for the 12 volunteers. It can be seen from this figure that, whilst lipid-bound XIIa is found in all of the samples tested, there is considerable variation in levels between individuals.

Example 2

In this example the existence of lipid bound activated Factor XII in plasma was demonstrated by addition to blood plasma of 2/215 antibody fragments labelled with a radiotracer (Iodine 125), precipitating the lipoproteins, and assessing the amount of radioactivity associated with the precipitated lipoprotein fraction.

Fab antibody fragments of antibody 2/215 were prepared using an "Immunopure Fab Preparation Kit" (Pierce, 3747 N Meridian Road, PO Box 117, Rockford, IL 61105) according to the manufacturer's instructions. These Fab Fragments were then radiolabelled with Iodine 125 by Amersham Pharmacia

Biotech(Pollards Wood, Nightingales Lane, Chalfont St Giles, HP8 4SP United Kingdom).

Citrated plasma was obtained from 64 patients admitted to hospital with chest pain.

5 μl of radiolabelled antibody was added to 1ml of citrated whole blood from each of the patients. After incubation for 3 hours, the plasma was centrifuged at 16,000g for 10 minutes to remove cellular components. lipoproteins were precipitated by the addition to 200 μl of plasma supernatant of 500 μl of a precipitating reagent containing 51.54 mM phosphotungstic acid, 0.07 M $MgCl_2$ adjusted to pH 6.15 with NaOH. After mixing, and incubating for 10 minutes samples were centrifuged at 16,000g for 10 minutes. The supernatant was removed, and the lipoprotein pellet was washed (to remove any residual aqueous phase activated Factor XII), by resuspending the pellet in 1ml of the precipitation reagent, centrifuging at 16,000g for 10 minutes and removing the supernatant. After performing this wash procedure three times, radioactivity associated with the pelleted material was measured using a single-well scintillation counter (Lab Logic, St John's House, 131 Psalter Lane, Sheffield, England S11 8UX).

Figure 2 shows the lipid bound XIIa levels obtained for the 64 patients. It can be seen from this figure, that whilst lipid-bound XIIa is found in all of the samples tested, there is considerable variation in levels between individuals.

Example 3

In this example, a microtitre ELISA immunoassay was used to demonstrate the presence of lipid bound activated Factor XII. Lipoproteins in plasma samples were captured by a antibody directed against a protein present on lipoprotein particles.

The presence of activated Factor XII on these lipoproteins was then demonstrated by the addition of alkaline phosphatase labelled 2/215 antibody.

Citrated plasma was obtained from 8 healthy volunteers,

100 µl aliquots of citrate plasma were added to wells of a microplate precoated with a goat polyclonal antibody against β-lipoprotein (Sigma, The Old Brickyard, New Road, Gillingham, Dorset, UK). After incubation for 60 minutes, the plates were washed with a borate buffered saline wash solution (pH 7.4). 100 µl of a conjugate containing alkaline phosphatase labelled 2/215 antibody was added to each well, and the plate was incubated for a further 60 minutes. After washing the plate again, 100 µl of phenolphthalein phosphate substrate was added. After a 30 minute incubation period, an alkaline Stop solution was added to inhibit further substrate conversion, and the absorbance was recorded at 550nm.

Figure 3 shows the lipid bound XIIa levels, as assessed by the ELISA method described above, obtained for the 8 volunteers. It can be seen from this figure, that whilst lipid-bound XIIa is found in all of the samples tested, there is considerable variation in levels between individuals.

CLAIMS:

1. A method which comprises detecting or determining lipid bound activated XII in a sample comprising tissue or a body fluid obtained from a mammalian subject.

2. A method as claimed in claim 1, where an assay has specifity for measuring lipid bound activated Factor XII, over other forms of activated Factor XII.

3. A method as claimed in claim 1 or claim 2, wherein a chromogenic assay is used to detect lipid bound activated Factor XII.

4. A method as claimed in claim 1 or claim 2, wherein an immunoassay is used to detect lipid bound activated Factor XII.

5. A method as claimed in claim 4, wherein the sample is contacted with a labelled antibody that is capable of binding to one or lipid bound activated Factor XII and any resulting an antigen-antibody complex is detected or determined.

6. A method as claimed in claim 5, wherein the antibody is mAb 2/215, which is produced by hybridoma 2/215, deposited at the European Collection of Animal Cell Cultures, Divisional of Biologics, PHLS Centre for Applied Microbiology and Research, Porton Down, Salisbury SP4 0JG, England (known as ECACC) under the deposit number 90011606, or is another monoclonal antibody having the same or similar XIIa binding properties for lipid-bound Factor XIIa as mAb 2/215.

7. A method as claimed in any one of claims 1 to 6, wherein the sample is a sample of a body fluid.

8. A method as claimed in claim 7, wherein the body fluid is whole blood or plasma.

9. A method of diagnosing or monitoring a disease or disorder in a subject, in which disease or disorder the amount of lipid bound activated Factor XII differ from those in a subject not having the disease or disorder, which comprises determining lipid bound activated Factor XII in a sample comprising body fluid obtained from the subject under investigation.

10. A method as claimed in claim 9, which comprises comparing the level of lipid bound activated Factor XII with levels of lipid bound activated Factor XII in a sample obtained from a subject not having the disease or disorder.

11. A method as claimed in claim 9 or claim 10, wherein the disease or disorder is a disease or disorder of the coagulation system.

12. A method as claimed in claim 9 or claim 10, wherein the disease or disorder is associated with inflammation or the inflammatory response.

13. A method as claimed in claim 9 or claim 10, wherein the disease or disorder is sepsis.

14. A method as claimed in claim 9 or claim 10, wherein the disease or disorder is acute or chronic inflammation, shock of different aetiologies, diabetes, allergy, a thrombo-

haemorrhagic disorder, an oncological diseases, or a cardiovascular condition.

15. A method as claimed in claim 9 or claim 10, wherein the disease or disorder is a myocardial infarction, acute coronary syndrome, angina, or thromboembolism

16. A method as claimed in claim 9 or claim 10, wherein the disease or disorder is spontaneous abortion.

17. A method as claimed in any one of claims 9 to 16, wherein lipid bound activated Factor XII is determined by a method as claimed in any one of claims 1 to 8.

18. A monoclonal antibody having the same or similar activated Factor XIIa binding properties as mAb 2/215, other than mAb 2/215.

19. A method for producing a monoclonal antibody that binds to lipid bound activated Factor XII which comprises raising monoclonal antibodies against Factor XII or a fragment thereof and screening the antibodies against lipid bound activated Factor XII

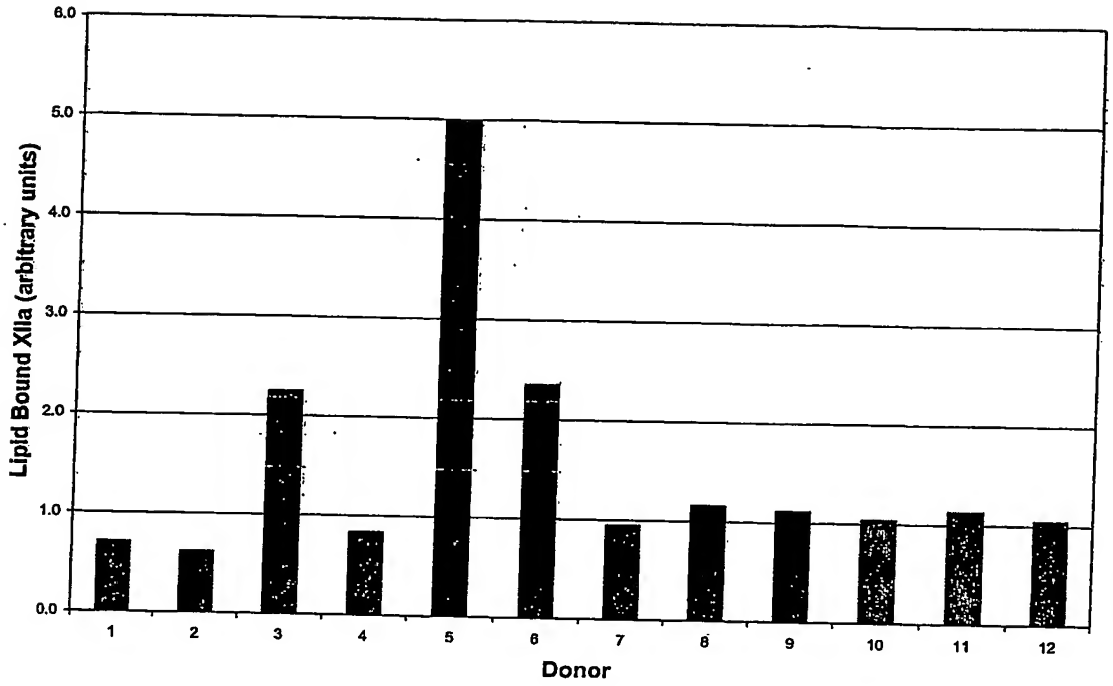
20. A method as claimed in claim 19, wherein one or more specific forms of Factor XIIa is used to raise the antibodies and are used for screening.

21. A method as claimed in claim 19 or claim 20, wherein mAb 2/215 is used as a reference antibody in screening.

ABSTRACT

ACTIVATED FACTOR XII VARIANT

Detection or determination of lipid bound activated Factor XII are useful as an aid to diagnosing or monitoring diseases and disorders in which the amount of lipid bound activated Factor XII is different from that in healthy subjects. Changes in the level of lipid bound activated Factor XII may indicate, for example, changes in the coagulation system. Changes in level may be associated with inflammation or the inflammatory response.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2.0
1.0
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Lipid Bound XIIa (arbitrary units)
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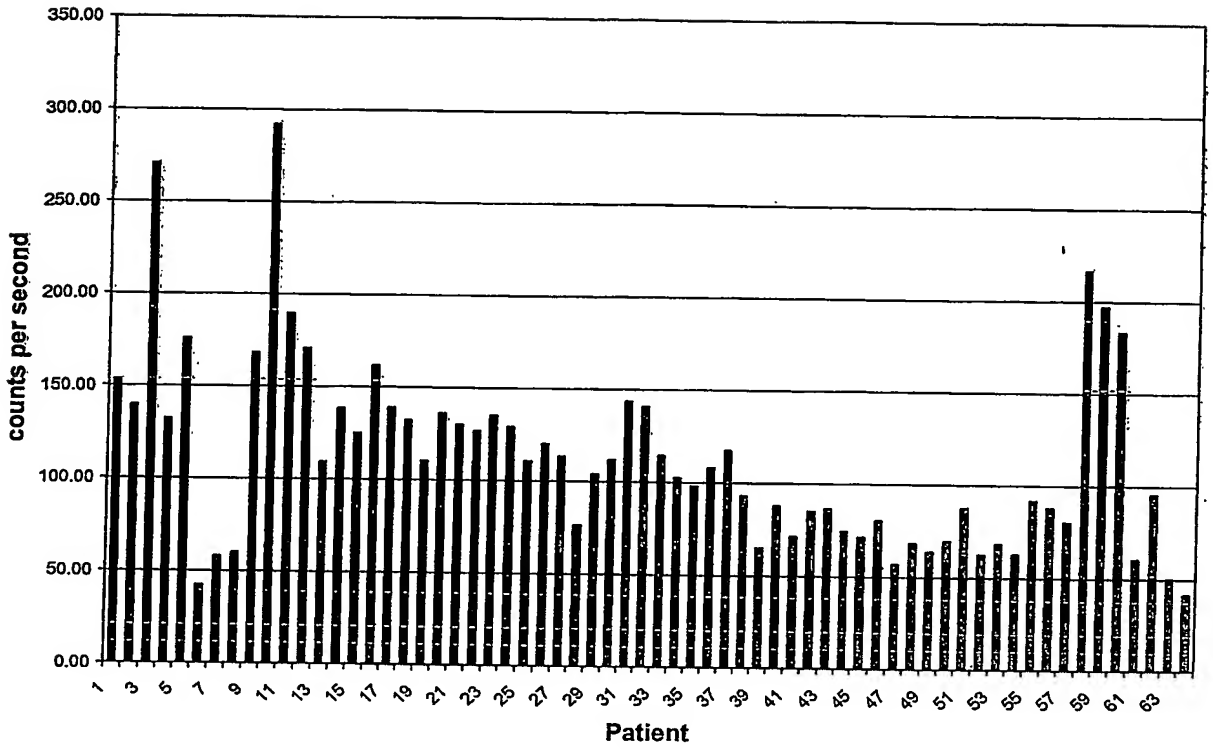
2
3
4
5
6
7
8
9
10
11
12
Donor

Figure 1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23
25
27
29
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41
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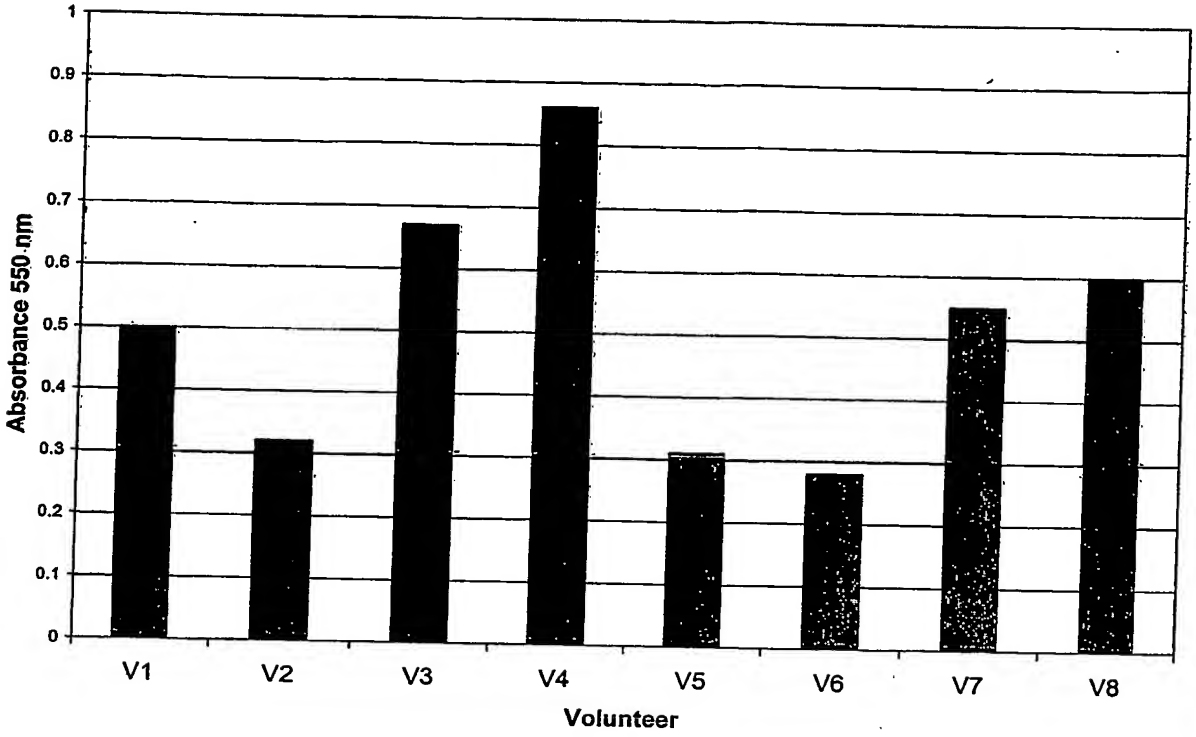
49
51
53
55
57
59
61
63
Patient

Figure 2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0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
V1
V2
V3
V4
V5
V6
V7
V8
Volunteer

Figure 3